Genetic transformation of *Pinus taeda* by particle bombardment

TANG Wei, Vanessa Samuels

(North Carolina State University, Forest Biotechnology Group, Centennial Campus P.O. Box 7247, Raleigh, NC 27695-7247, USA)

Abstract: A protocol is presented for genetically engineering loblolly pine (*Pinus taeda* L.) using particle bombardment. This protocol enabled the routine transformation of loblolly pine plants that were previously difficult to transform. Mature zygotic embryos were used to be bombarded and to generate organogenic callus and transgenic regenerated plants. Plasmid pB48.215 DNA contained a synthetic Bacillus thuringiensis (B.t.) cryIAc coding sequence flanked by the double cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (Nos) terminator sequences, and the selectable marker gene, neomycin phosphotransferase II (nptII) controlled by the promoter of the nopaline synthase gene was introduced into loblolly pine tissues by particle bombardment. The transformed tissues were proliferated and selected by kanamycin resistance conferred by the introduced NPTII gene. Shoot regeneration was induced from the kanamycin-resistant callus, and transgenic plantlets were then produced. The presence of the introduced genes in the transgenic loblolly pine plants was confirmed by polymerase chain reactions (PCR) analysis, by Southern blot analysis, and insect feeding assays. The recovered transgenic plants were acclimatized and then established in soil.

Keywords: *Pinus taeda* L.; Biolistic transformation; *Bacillus thuringiensis* (B.t.) cryIAb; Insect feeding bioassay

Abbreviations: BA benzyladenine; B.t. *Bacillus thuringiensis*; CaMV cauliflower mosaic virus; 2,4-D 2,4-dichlorophenoxyacetic acid; IBA indole-3-butyric acid; Nos nopaline synthase; nptII neomycin phosphotransferase II gene; PCR polymerase chain reactions

CLC: Q55; S791.255.04 **Document code**: A **Article ID**: 1007-662X(2002)02-0091-07

Introduction

There is substantial interest in the genetic improvement of pine. Genetic modification of pine for insect resistance, disease and stress resistance, lignin content, and the improvement of wood quality is thus of major commercial importance. Pine improvement by conventional plant breeding methods has been limited mainly due to the prohibitively long reproduction cycles and slow seed maturation of these plants. Therefore, the application of genetic engineering techniques to pine improvement appears to be an attractive alternative. Although transformation protocols are available for pine (Humara *et al.* 1999; Tzfira *et al.* 1996), there is no report available on the stable genetic transformation of loblolly pine by particle bombardment. Among gene transfer methods reported, the biolistic technique appears to be the best technique for gene transfer into forest tree species. Since transgenic plants from microprojectile-mediated gene transfer have been reported for rice (Alam *et al.* 1998; Christou *et al.* 1991; Data et al. 1998), Soybean (Christou *et al.* 1989), papaya (Fitch *et al.* 1990), potato (Perlak *et al.* 1993), tobacco (Iida *et al.* 1991), and poplar (Han *et al.* 1997; McCown *et al.* 1991), potential progress has been made in plant genetic transformation (Franche *et al.* 1997; Schuler *et al.* 1998). Transient expression and regeneration of transgenic conifers via *Agrobacterium*-mediated transformation have been reported for *Pinus pinea* (Humara *et al.* 1999), *Pinus halepensis* (Tzfira *et al.* 1996), *Larix decidua* (Huang *et al.* 1991), hybrid larch (Levee et al. 1997), and larch (Shin *et al.* 1994). Stable transformation of conifers using biolistics has been achieved for *Picea abies* (Walter *et al.* 1999), *Larix laricina* (Klimaszewska *et al.* 1997), *Picea glauca* (Ellis *et al.* 1993), and *Pinus radiata* (Walter *et al.* 1998). Loblolly pine is an important forest species in the tropical and subtropical region. We conducted a number of studies to develop a stable gene transfer system for loblolly pine using particle bombardment. We studied several factors involved in particle bombardment-mediated transformation. Those factors included bombardment conditions, genotypes, pre-culture conditions, and selection. Here, we report a reproducible method for the gene transfer and regeneration of transformed plants from the loblolly pine mature zygotic embryos using particle bombardment. A key to use mature zygotic embryos of different genotypes of loblolly pine as targeting tissues to develop a optional genetic transformation protocol. In this investigation, we reported the work on transgenic loblolly pine plants with enhanced resistance to pine moth (*Dendrolimus punctatus* Walker) and *Crypyothelea formosicola* Staud.

Materials and methods

Plant materials

Mature seeds of loblolly pine were collected from Shaoyang Loblolly Pine Seed Orchard (genotypes Hb Ma, and

Biography: TANG Wei (1964-), male, Ph. Doctor. Research associate, North Carolina State University, Forest Biotechnology Group, Centennial Campus P.O Box 7247, Raleigh, NC 27695-7247, USA. E-mail: wei_tang@ncsu.edu

Received date: 2001-08-08

Responsible editor: Chai Ruihai

Mc), Hunan Province, China, and the Southeast America (genotypes J-56 and S-1003, provided by Professor Weihua Zhong) in October 1993-1996, and stored in plastic bags at temperature of 4 °C before they were used to tissue culture. Seeds were disinfected by immersion in 70% w/w ethanol alcohol for 30 s and in 0.1% mercuric chloride for 10-15 min, followed by four to five rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from the megametophytes and placed horizontally on a solified callus induction medium in flasks or Petri dishes. Mature zygotic embryo explants were used to transformation experiments after cultured on pretreatment medium consisted of TE medium (Tang *et al.* 1998) supplemented with 10 $mg \cdot L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D), 4-mg/L benzyladenine (BA), and 4 $mg \cdot L^{-1}$ kinetin for 1-3 weeks.

Plasmid construction

Plasmid pB48.215 DNA contained a synthetic *Bacillus thuringiensis* (B.t.) crylAc coding sequence flanked by the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (Nos) terminator sequences, and the selectable marker gene, neomycin phosphotransferase II (nptII) controlled by the promoter of the nopaline synthase gene (Li *et al.* 1994). The double 35 S promoter and translation enhancer fragments cut by Hind III and BamHI from pD511 were cloned to pBin437 to produce pBin438. The *Bacillus thuringiensis* (B.t.) crylAc gene fragments cut by BamHI and SalI from pB48.103 were inserted in pBin438 to produce pB48.215 (Li *et al.* 1994). Plasmid pB48.215 were transferred into E coli strain DH5α and bacteria were cultured on LB medium with 100mg/L carbenicillin and 50mg/l kanamycin. Plasmid pB48.215 were largely isolated from bacterium cultures grown overnight at 37 °C in liquid LB medium (Sambrook *et al.* 1989) supplemented with 100 $mg \cdot L^{-1}$ carbenicillin and 50 $mg \cdot L^{-1}$ kanamycin and purified by CsCl gradient centrifuge, and then used to biolistic transformation.

Particle bombardment

Microprojectile bombardment was carried out using the Biolistic PDS 1000/He System (Bio-Rad, Hercules, Calif.) at 1100psi following the manufacturer's instructions and protocol. All accessible parts of this equipment were surface-sterilized using 70% ethyl alcohol 15 min before use. Microcarriers were prepared as follows: 3-mg gold (0.6, 1.0, and 1.1μm) particles, suspended in 50-μl distilled water, were mixed with 20-μg DNA of plasmid, followed by addition of 175-μL CaCl2 (2.5M) and 70-μL spermidine (0.1M, Sigma). The mixture was gently vortexed for 10 mix at room temperature and then centrifuged briefly. The gold particles were washed twice with 100% ethanol and resuspended in 100% ethanol (50 μl). Fifteen microliters microcarrier suspension was used for each bombardment. Explants were placed on the TE callus induction medium for 28 h before bombardment. Bombardment was performed with a rupture disk pressure of 1100, 1350, and 1550 psi, respectively, a gap distance of 1.5 cm, a macrocarrier travel distance of 8 mm, and target distance of 12 cm. After bombardment, embryos were transferred to TE medium containing 15 $mg \cdot L^{-1}$ kanamycin before being assessed for activity of introduced genes. Calli were subcultured every 14 days. Kanamycin-resistant calli were maintained on selection medium for longer than was practically required for regeneration, to ensure that a true frequency for kanamycin resistance was established. Approximately 1-μg DNA was introduced per bombardment. Tissues of all three genotypes were bombarded with plasmid. Similar samples of all three species that were not bombarded and bombarded with gold particles without DNA served as the controls. Treatments were replicated three times. Then bombarded mature zygotic embryos were transferred onto callus induction medium supplemented with 500 $mg \cdot L^{-1}$ carbenicillin and 15 $mg \cdot L^{-1}$ kanamycin. After three weeks, mature zygotic embryos were transferred to fresh callus induction medium.

Selection and regeneration

After organogenic calli were initiated from transformed mature zygotic embryos cultured on induction medium with 15 $mg \cdot L^{-1}$ kanamycin, Plant regeneration was carried out on differentiation supplemented with selection pressure according to a procedure previously described (Tang *et al.* 1998). Differentiation was evaluated by the percentage of calli forming transgenic adventitious shoots. All media were supplemented with 3 % sucrose and 0.3% Phytagel (Sigma). Media were adjusted to pH 5.8 prior to autoclaving 20 min at 121 °C. All cultures were cultured at 25 °C culture room. Adventitious shoot induction was conducted in the dark and adventitious shoot differentiation and proliferation and rooting were conducted at 25 °C under a 16-h photoperiod with cool fluorescent light (100 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After acclimatization of regenerated plantlets was conducted by decreasing relative humidity to ambient condition over a period of one week, plantlets were established in soil. The frequency of embryos forming transgenic calli and the frequency of transgenic calli forming transgenic plants were determined in the 9th week of culture. The data are analyzed by the Analysis of Variance (ANOVA).

Molecular analysis

DNA was extracted from putative transgenic plants using the Boehringer Mannheim plant DNA extraction kit. Polymerase chain reactions (PCR) were carried out in 1(PCR buffer, 200-μM dexoynucleotide triphosphates, 0.4-μM primer, 1 unit of Taq DNA polymerase (Boehringer Mannheim), and 100 ng of template DNA in 50μl reaction volume. The primers used were sequence between -55 and -78 of 35S promoter and sequence between 346 and 363 of the B.t. gene. The reaction consisted of 30 cycles of 1-min denaturation at 94°C, 45 sec annealing at 55 °C, and 1 min elongation at 72°C. Program begun with 4 min at 94 °C

and ended with a 10-min elongation at 72 °C. PCR products were separated on 0.8% mass/volume agarose electrophoresis gels at 80V for 1 h, stained with ethidium bromide at a concentration of 0.5 $\mu g \cdot mL^{-1}$ and captured using Eagle Eye video imaging system (Stratagene, La Jolla, Calif.). For Southern bolting analysis, DNA (20 μg) from putative transgenic and non-transformed control plants was digested with overnight with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) at 37 °C. Digested DNA of each line were separated through a 1% agarose gel prepared in 1×TBE (Sambrook *et al.* 1989) and blotted onto Hybond N+ membrane (Amersham). The DNA fixed on membranes was hybridized (at 65 °C) with the Bacillus thuringiensis (B.t.) crylAc gene probe (BamHI and SacI (Boehringer Mannheim) fragment of B.t. gene), which was labeled with 32P-dCTP (Ready to Go Labeling Beads (Pharmacia)). Hybridization was performed according to Sambrook *et al.* (1989) in 5×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 $mg \cdot L^{-1}$ denatured fish sperm, 0.1 $g \cdot mL^{-1}$ dextran sulfate for 20 h before membranes were washed with 0.1×SSPE, 0.1% SDS solution at 65 °C. Hybridizing bands were detected by 5 days exposure to Kodak X-OMAT AR autoradiography films at –80 °C for three days.

Scanning electron microscopy

Samples were prepared for scanning electron microscopy at the different stage of in vitro regeneration. Tissues were fixed overnight in 4% glutaraldehyde and 100 mM phosphate buffer (pH 7.0), washed one time in 100 mM phosphate buffer (pH 7.0) for 30 min, followed by dehydration in successive ethanol solutions of 85%, 95%, and 100%, each repeated twice for 5 min. Specimens were dried in a critical-point-drier with CO_2 for 2 h, mounted on Cu stubs and gold-coated. The samples were examined and photographed in HITACHI S-800 scanning electron microscope.

Insect bioassay

All positive transgenic plants expressing the Cry IAc protein were tested for lethality and growth retardation of Dendrolimus punctatus Walker and Crypyothelea formosicola Staud larvaes using a whole plant-feeding assay. The test plants were individually infested with larvaes of 3-5 mm in length for seven days at 25 °C under 16/8 h light/dark regime. Transgenic plants were placed on a piece of moistened filter paper in a petri dish (100×200 mm). Larvae were placed on the shoot, and the petri dish was sealed with masking tape. Incubation was done for seven days at 25 °C. The bioassay was replicated three times for each plant. After seven days, the number of alive and dead larvae was determined for each petri dish. A similar infestation was also done for control plants. The mortality rate was expressed as a percentage (the number of dead larvae/number of larvae applied) × 100%. The data is analyzed by the Analysis of Variance (ANOVA)

Results and discussion

Transformation and selection of transformants

We used mature zygotic embryos of loblolly pine as target tissues of particle bombardment. Actively dividing cells in embryos provided a window of competence for transformation. Tobacco cells bombarded at the M- and G2-phases have 4-6 times higher transformation efficiencies than those at the S- and G1-phases (Iida *et al.* 1991). T-DNA integrates preferentially into sequences that can potentially be transcribed (Perlak *et al.* 1991). Studies on the integration of T-DNA into the plant genome strongly suggest that host DNA synthesis is required (Gheysen *et al.* 1987). Thus, the greater the number of actively dividing cells in the tissue being bombarded, the higher probability of obtaining stable expression in the bombarded tissue. In the present protocol it was critical that the embryos were bombarded after the tissue was pre-cultured in TE medium for a time at least sufficient to induce cell division on the target tissue. Cells hit by the DNA-coated particles at this stage were likely to be the cells from which proliferate calli are derived. Ellis *et al.* (1993) observed that the percentage of spruce somatic embryos expressing the GUS gene after bombardment increased progressively with advanced developmental stages. The transformed developed on the surface of the embryos. Among three different sizes of gold particles (0.6, 1.0, and 1.1 mm), the smallest particles were most efficient in delivering the genes and different levels of helium gas pressures (1 100, 1 350, and 1 550 pounds per square inch) did not affect transformation efficiency (data not shown).

Bombarded embryos were first induced to form calluss by being incubated in a medium without kanamycin for 28 days. The use of the pre-culture on TE callus induction medium for 14 days produced significantly higher number of callus than did the control. The initial 30 days of culture without kanamycin selection was intended to promote active proliferation of callus on the surface of the bombarded embryos. The proliferated calli are then induced to regenerate shoots under kanamycin selection. The optimal concentration for selecting transformed cells and tissues was determined by culturing mature zygotic embryos on callus induction medium containing different concentration of kanamycin. According to the preliminary experiments, 15-$mg \cdot L^{-1}$ kanamycin was used to identify transformed cells and tissues. Two to three weeks after mature zygotic embryos were transferred onto callus induction medium supplemented with 15-$mg \cdot L^{-1}$ kanamycin, mature zygotic embryos began to form calli. Callus was formed on cotyledons, hypocotyl, and radicles of embryos two to three weeks after bombardment. Proliferation of transgenic calli was achieved by sub-cultured kanamycin resistant calli on fresh callus induction medium with kanamycin. This sequential manipulation was critical for transformation success as prior attempts to obtain transformants from embryos trans-

ferred directly to TE medium containing kanamycin after bombardment.

Differentiation of transgenic calli and plant regeneration

The frequency of embryos forming transgenic calli (Fig. 1) and the frequency of calli forming transgenic plantlets (Fig. 2) were influenced by the genotypes of loblolly pine. The frequency of putative transgenic calli ranged from 3.2% (genotype Hb) to 28.7% (genotype J-56) (Fig. 1). Putative transgenic adventitious shoots (Fig. 3a) were formed on the surface of kanamycin resistant calli. Nine to twelve weeks after kanamycin-resistant calli were transferred to differentiation medium. The frequency of adventitious bud formation ranged from 1.8% (genotype Hb) to 14.6% (genotype J-56) on the differentiation medium supplemented with BA and indole-3-butyric acid (IBA) in the 9th week of culture (Fig. 2). Root primordia was usually formed at the base of shoots (Fig. 3b). Elongation of transgenic shoots (Fig. 3c), rooting of transgenic shoots (Fig. 3d), and acclimatization of regenerated plantlets were carried out according to a procedure previously described (Tang et al. 1998). Rooting frequency 5.3%-17.9% was observed and both growth and phenotype of regenerated plantlets appeared similar to the untreated control. Sixteen regenerated plantlets from transgenic organogenic calli of loblolly pine were transferred from culture flasks into a perlite:peatmoss:vermiculite (1:1:1 v/v/v) soil mixture, and seven acclimatized plantlets were successfully established in the field (Fig. 3e).

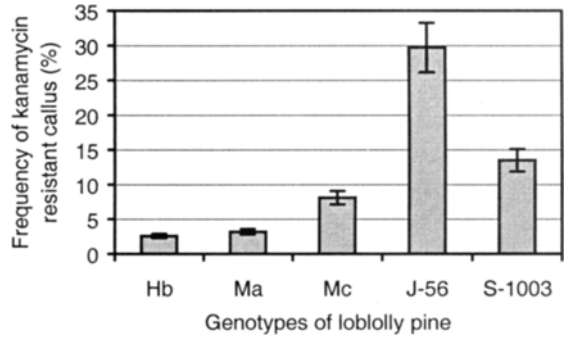

Fig. 1 Influence of genotypes on the frequency (%) of embryos forming kanamycin resistant callus in loblolly pine. (Experiments were repeated three times and each replicate consisted of 30 embryos. The frequency of embryos forming kanamycin resistant calli was determined in the 9th week of culture. Values represent the means ± S.D.).

Confirmation of T-DNA integration

Trangenic plantlets from independent transformation events were analyzed by PCR and Southern hybridization. Genomic DNA was isolated from control and putative transgenic plants using the Boehringer Mannheim plant DNA extraction kit. PCR analysis showed that predicted bands were amplified from transgenic plants, but not from the control (Fig. 3f). Twenty micrograms of DNA was digested overnight with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) at 37 °C and was used in Southern hybridization experiments. No bands were detected in non-transformed control plants, whereas bands were observed in transgenic plants (Fig. 3g). These results confirm that the foreign genes have been integrated into the *Pinus taeda* genome. The Southern results of regenerated transgenic plants demonstrated that one to two bands that represented junctions between T-DNA and adjacent plant DNA. These findings show that these are transformants in which the insert DNA containing B.t. gene has integrated at one to two sites in the plant genome. The integration of foreign genes in regenerated transgenic plants was also observed in *Pinus radiata* via particle bombardment (Walter *et al.* 1998).

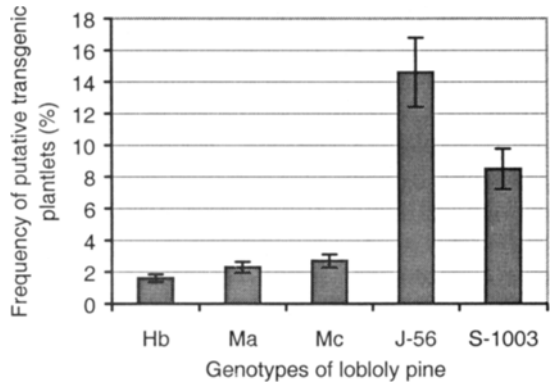

Fig. 2 Influence of genotypes on the frequency (%) of embryos forming kanamycin resistant callus in loblolly pine. (Experiments were repeated three times and each replicate consisted of 30 embryos. The frequency of embryos forming kanamycin resistant calli was determined in the 9th week of culture. Values represent the means ± S.D.).

Insect bioassay

Insect resistance of transgenic plants with introduced synthetic *Bacillus thuringiensis* crylAb and crylAc genes have been confirmed by insect bioassay (Cheng *et al.* 1998; Stewart *et al.* 1996; Xiang *et al.* 2000). In this investigation, the transgenic calli and regenerated plants expressing the B.t. toxin protein were selected for insect bioassay. Insecticidal activity was evaluated by placing larvae of *Dendrolimus punctatus* Walker and *Crypyothelea formosicola* Staud on the test transgenic calli (Table 1) and transgenic plants (Table 2), which were confirmed by PCR and Southern blot. We observed that the larvae often bores small holes on the first or second day of feeding on the regenerated pants positively identified by PCR and Southern blot analysis. Two days later, some larvae died and the others ceased feeding and became stunted, depending the age of the larvae. However, the larvae on the non-transformed control plants and susceptible plants continued to feed and grew well to more than 0.9 cm in length and green in appearance after seven days of feeding.

Eventually these larvae pupated later without mortality, leaving behind severely damaged plants.

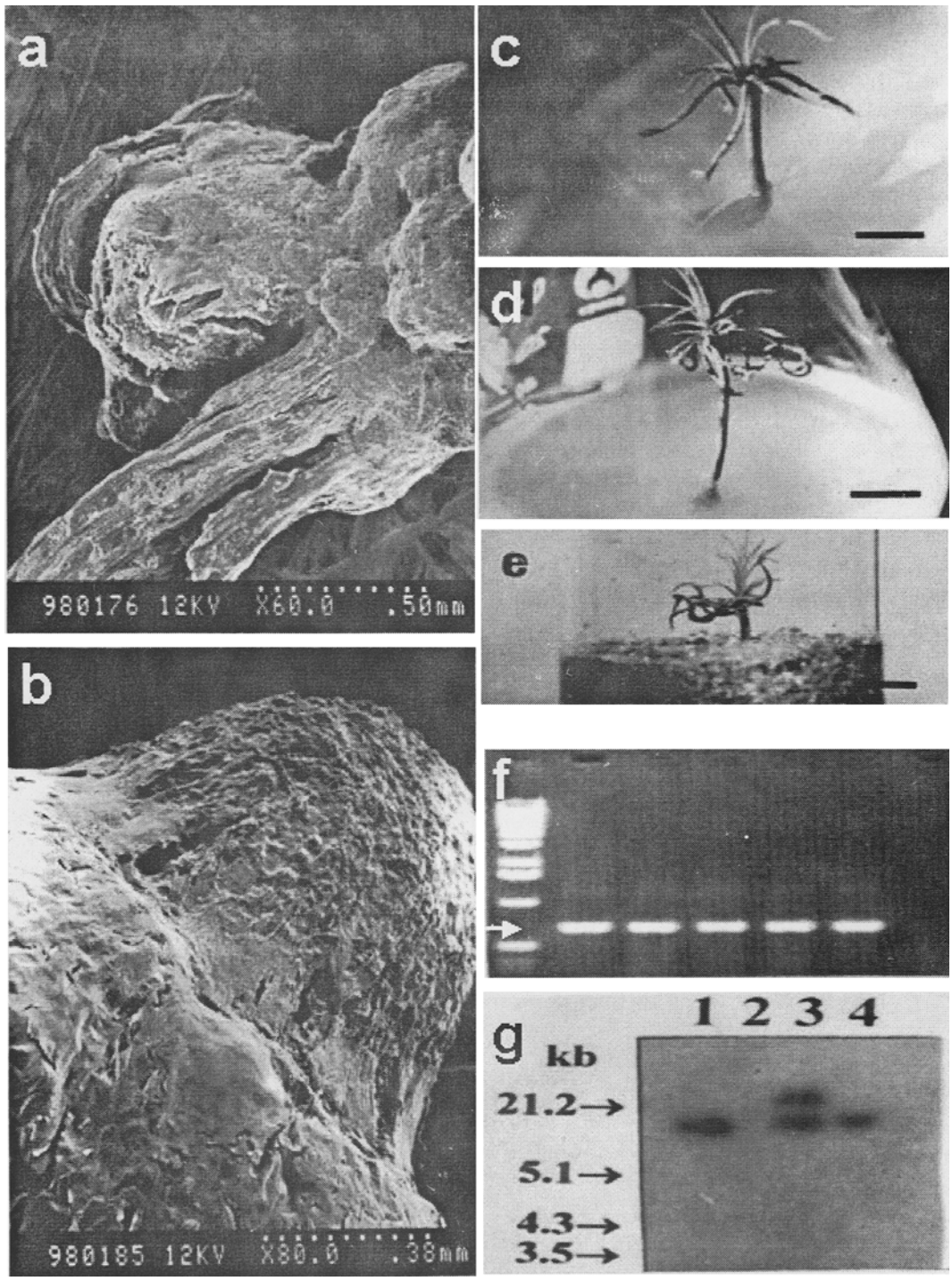

Fig. 3 Particle bombardment-mediated transformation and molecular analysis of transgenic plant in loblolly pine (genotype J-56). Fig. 3(a) shows the transgenic shoots from organogenic callus, Fig 3(b) shows the root primdria from transgenic shoot; Fig 3(c) shows the elongation growth of kanamycin-resistant adventitious shoots (Bar = 0.5 cm); Fig 3(d) shows the rooting of transgenic shoot (Bar = 0.5 cm); Fig. 3(e) shows the transgenic regenerated plantlet established in soil (Bar = 1 cm). Fig. 3(f) shows PCR analysis of transgenic plants, predicted 520bp DNA fragment (arrow) were amplified from transgenic plants (lane 3-6) by specific primers, but not from the control (lane 7) Lane 2 plasmid DNA control, lane 1 1 kb DNA ladder; Fig. 3(g) shows the southern blot analysis of transgenic loblolly pine plants DNA was digested with the restriction enzyme EcoRI and Hind III (Boehringer Mannheim) overnight at 37°C, was hybridized (at 65°C) with the B t. probe (BamHI-SacI fragment of B.t gene), which was labeled with 32P-dCTP (Ready to Go Labeling Beads (Pharmacia), lane 1 plasmid DNA of pB48.215 control (5pg), lane 2 DNA from non-transformed plant of genotype J-56 (20 μg), lane 3-4 DNA from transgenic plants of genotype J-56 (20 μg).

Through extensive evaluations of factors involved in the particle bombardment-mediated transformation, we developed the present protocol with which a large number of stably transformed loblolly pine plants were generated. This protocol, with slight modification if necessary, appears applicable to all loblolly pine genotypes and other coniferous species that have been recalcitrant to transformation. This protocol could be useful to genetic engineering of the commercially important loblolly pine clones with genes conferring virus resistance, disease resistance, stress tolerance, wood property, lignin contents, and other economical qualities.

Table 1 Insect resistance analysis of transgenic callus from different genotypes of loblolly pine

Genotypes	Morality of insect (%)			
	Dendrolimus punctatus	Control	*Crypyothelea formosicola*	Control
Hb	48.6±4.5	8.2±1.1	59.1±4.3	8.7±0.8
Ma	44.9±3.7	9.7±1.3	62.7±3.6	9.7±1.1
Mc	42.1±4.3	9.1±1.2	74.8±6.7	9.8±1.3
J-56	57.9±4.8	8.8±1.8	55.6±7.9	9.6±1.4
S-1003	48.5±5.3	9.3±1.5	85.3±8.4	9.2±1.1

Experiments were repeated three times and each replicate consisted of 30 insects. The morality of insect was determined in the 7th day of insect bioassay. Values represent the means ± S.D.

Table 2 Insect resistance analysis of transgenic regenerated plants from different genotypes of loblolly pine

Genotypes	Morality of insect (%)			
	Dendrolimus punctatus	Control	*Crypyothelea formosicola*	Control
Hb	50.7±3.5	8.5±1.2	52.3±4.3	7.8±1.8
Ma	51.3±4.7	8.7±2.3	52.7±3.8	8.7±1.5
Mc	51.9±5.3	8.1±1.5	54.8±6.9	8.9±1.3
J-56	56.9±4.7	9.2±1.8	65.6±7.9	9.4±1.8
S-1003	53.5±5.2	9.3±1.5	86.3±8.4	9.9±1.7

Experiments were repeated three times and each replicate consisted of 30 insects. The morality of insect was determined in the 7th day of insect bioassay. Values represent the means ± S.D.

Acknowledgements

We wish to thank Professor C. Koncz, Professor R. Martienssen, and Professor Y.C. Tian for encouragement and support.

References

Alam M.F, Datta, K., Abrigo, E, Vasquez, A., Senadhira, D., Datta, S.K. 1998. Production of transgenic deepwater indica rice plants expressing a synthetic *Bacillus thuringiesis cryIA*(b) gene with enhanced resistance to yellow stem borer [J]. Plant Sci., 135: 25-30.

Cheng, X.Y, Sardana, R., Kaplan, H., Altosaar, I. 1998. Agrobacterium-transformed rice plants expressing synthetic cry1Ab and cry1Ac genes are highly toxic to striped stem borer and yellow stem borer [J]. Proc Natl Acad Sci USA, 95: 2767-2772.

Christou, P., Ford, T., Kofron, M. 1991. Production of transgenic rice (*Oriza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos [J]. Bio/Technology, 9: 957-962.

Christou P, Swain, W., Yang N-S, McCabe, D. 1989. Inheritance and expression of foreign genes in transgenic soybean plants [J]. Proc Natl Acad Sci USA, 86: 7500-7504.

Datta, K., Vasquez, A., Tu, J., Torrizo, L., Alam, M.F., Oliva, N., Abrigo, E., Khush, G.S., Datta, S.K. 1998.Constitutive and tissue-specific differential expression of cryIA(b) gene in transgeic rice plants conferring enhanced resistance to rice insect pest [J]. Theor. Appl. Genet., 97: 20-30.

Ellis, D.D., McCabe, D.E., McInnis s, Ramachandran, R., Russell, D.R., Wallace, K.M., Martinell, B.J., Roberts, D.R., Raffa, K.F., McCown, B.H. 1993. Stable transformation of Picea glauca by particle acceleration [J]. Bio/Technology, 11: 84-89.

Fitch, M., Manshardt, R., Gonsalves, D., Slightom, J., Sanford, J. 1990. Stable transformation of papaya via microprojectile bombardment [J]. Plant Cell Rep., 9:189-194.

Franche, C., Diouf, D., Le, Q., Bogusz, D., N'Diaye, A., Gherbi, H., Gobe, C., Duhoux, E. 1997. Genetic transformation of the actinorhizal tree Allocasuarina verticillata by Agrobacterium tumefaciens [J]. Plant J., 11: 897-904.

Gheysen, G., van Montagu, M., Zambryski, P. 1987. Integration of *Agrobacterium tumefaciens* T-DNA involves rearrangements of target plant DNA sequences [J]. Proc Natl Acad Sci USA, 84: 9006-9010.

Han, K-H, Ma, C., Strauss, S.H. 1997. Matrix attachment regions enhance transgene expression and transformation in poplar [J]. Transgen Res., 6:415-420.

Huang, Y., Diner, A.M., Karnosky, D.F. 1991 *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix deciduas* [J]. In Vitro Cell Dev Biol., 27: 201-207.

Humara, J.M., Lopez, M., Ordas, R.J. 1999. *Agrobacterium rhizogenes*-mediated transformation of Pinus pinea L. cotyledons: an assessment of factors influencing the efficiency of uidA gene transfer [J]. Plant Cell Rep., 19: 51-58.

Lida, A., Yamashida, T., Yamada, Y., Morikawa, H. 1991. Efficiency of particle-bombardment-mediated transformation is influenced by cell stage in synchronized cultured cells of tobacco. Plant Physiol., 97:1585-1587.

Klimaszewska, K., Devantier, Y., Lachance, D., Lelu, M.A., Charest, P.J. 1997. *Larix laricina* (tamarack): somatic embryogenesis and genetic transformation [J]. Can J. For. Res., 27: 538-550.

Levee, V., Lelu, M.A., Jouanin, L., Cornu, D., Pilate, G. 1997. Agrobacterium tumefaciens-mediated transformation of hybrid larch (*Larix kaempferi* × *L. decidua*) and transgenic plant regeneration [J]. Plant Cell Rep., 16: 680-685.

Li, T.Y., Tian, Y.C., Qing, X.F. 1994. Studies on high-efficient insect resistance transgenic tobacco [J]. China Sinica. (B), 24: 276-268.

McCown, B.H., McCabe, D.E., Russell, D.R., Robison, D.J., Barton, K.A., Raffa, K.F. 1991. Stable transformation of *Populus* and incorporation of pest resistance by discharge particle acceleration [J]. Plant Cell Rep., 9: 950-954.

Perlak, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L., Fischoff, D.A. 1991. Modification of the coding sequence enhances plant expression of insect control proteins [J]. Proc Natl Acad Sci USA, 88: 3324-3328.

Perlak, F.J., Stone, T.b, Muskopf ,Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever D., Fischoff, D.A. 1993. Genetically improved potatoes: protection from damage by Colorado potato beetle [J]. Plant Mol Biol., **22**: 313-321.

Sambrook, J, Fritsch, E.F., Mamiatis, T. 1989. Molecular Cloning: a laboratory manual (2nd ed) [M]. New York: Cold Spring Harbor.

Schuler, TH., Poppy, G.M., Kerry, B.R., Denholm, L. 1998. Insect-resistant transgeic plants [J]. Trends Biotechnol., 16: 168-175.

Shin, D.I., Podila, G.K., Huang, Y., Karnosky, D.F. 1994. Transgenic larch expressing genes for herbicide and insect resistance [J]. Can. J. For. Res., 24: 2059-2067.

Stewart, CN. Jr, Adang, M.J., All, J.N., Boerma, H.R., Cardineau, G., Tucker, D., Parrott, W.A. 1996. Genetic transformation, recovery and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiesis cryIA*(c) gene [J]. Plant Physiol 112: 121-129.

Tang, W., Ouyang, F., Guo, Z..C. 1998. Plant regeneration through organogenesis from callus induced from mature zygotic embryos of loblolly pine [J], Plant Cell Rep., 17: 557-560.

Tzfira, T., Yarnitzky, O., Vainstein, A., Altman, A. 1996. *Agrobacterium rhizogenes*-mediated DNA transfer in *Pinus halepensis* Mill [J]. Plant Cell Rep., 16: 26-31.

Walter, C., Grace, L.J., Wagner, A., White, D.W.R, Walden, A..R, Donaldson, S..S., Hinton, H., Gardner, R.C., Smith, D.R. 1998. Stable transformation and regeneration of transgenic plants of *Pinus radiata* D.Don [J]. Plant Cell Rep., 17: 460-469.

Walter, C., Grace, L., Donaldson, S.S., Moody, J., Gemmell, J.E., van der Maos, S., Kvaalen, H., Lonneborg, A. 1999. An efficient Biolistic transformation protocol for *Picea abies* embryogenic tissue and regeneration of transgenic plants [J]. Can. J. For. Res., 29: 1539-1546.

Xiang, Y., Wong, W.K.R., Ma, M.C, Wong, R.S.C. 2000. *Agrobacterium*-mediated transformation of *Brassica campestris* spp. Parachinensis with synthetic *Bacillus thuringiensis* cry1Ab and cry1Ac genes [J]. Plant Cell Rep., 19:251-256.